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Relevance of neuronal and glial NPC1 for synaptic input to cerebellar Purkinje cells

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ABSTRACT

Niemann–Pick type C disease is a rare and ultimately fatal lysosomal storage disorder with variable neurologic symptoms. The disease-causing mutations concern NPC1 or NPC2, whose dysfunction entails accumulation of cholesterol in the endosomal–lysosomal system and the selective death of specific neurons, namely cerebellar Purkinje cells. Here, we investigated whether neurodegeneration is preceded by an imbalance of synaptic input to Purkinje cells and whether neuronal or glial absence of NPC1 has different impacts on synapses. To this end, we prepared primary cerebellar cultures from wildtype or NPC1-deficient mice that are glia-free and highly enriched with Purkinje cells. We report that lack of NPC1 in either neurons or glial cells did not affect the excitability of Purkinje cells, the formation of dendrites or their excitatory synaptic activity. However, simultaneous absence of NPC1 from neuronal and glial cells impaired the presynaptic input to Purkinje cells suggesting a cooperative effect of neuronal and glial NPC1 on synapses.

Introduction

Niemann–Pick type C disease (NPC; OMIM #257220) is a rare and ultimately fatal, autosomal recessive lysosomal storage disorder with diverse neurologic symptoms including ataxia and vertical supranuclear gaze palsy (Patterson et al., 2012). The disease is caused by mutations in the genes encoding for Niemann–Pick C1 protein (NPC1) or NPC2, whose dysfunction leads to accumulation of cholesterol and other lipids in the cellular endosomal–lysosomal system (Rosenbaum and Maxfield, 2011; Vance and Peake, 2011). A pathologic hallmark of NPC is the loss of specific types of neurons, namely cerebellar Purkinje cells (PCs), in human patients (Harzer et al., 1978) as well as in mouse (Higashi et al., 1993; Tanaka et al., 1988) and cat models of the disease (March et al., 1997). At present, it is unknown, why NPC1 deficiency causes type-specific neuronal degeneration.

Previous studies reported that NPC1 and NPC2 are located at synapses and in surrounding astrocytic processes (Hu et al., 2000; Karten et al., 2006; Ong et al., 2004; Xu et al., 2011). Neurodegeneration in NPC1-deficient mice and cats was found to start at nerve terminals, axons and dendrites and to progress retrogradely to neuronal somata (March et al., 1997; Ong et al., 2001; Sarna et al., 2003; Zervas et al., 2001). Prominent changes in the levels of different neurotransmitters were observed in the cerebellum of NPC1-deficient mice (Yadid et al., 1998). Together, these observations suggest that NPC1 dysfunction perturbs synaptic activity in PCs (Paul et al., 2004) in a cell-autonomous manner (Ko et al., 2005) or with a contribution from degenerating glial cells (German et al., 2002). To test these hypotheses, we took advantage of a glia- and serum-free cerebellar culture preparation from postnatal mice that is highly enriched with PCs thanks to antibody-based cell selection (Buard et al., 2010). These primary cultures allowed us for the first time to test, whether the lack of NPC1 in neurons or glial cells affects the level of synaptic activity in PCs. Our results show that the absence of NPC1 from either neurons or glial cells left the excitability of PCs, the formation of dendrites or their synaptic activity unaffected, whereas simultaneous NPC1 deficiency in both cell types impaired synaptic input to PCs, possibly at the presynaptic level.

Results

To study the relevance of NPC1 for synaptic activity in cerebellar PCs, we used a serum- and glia-free cerebellar culture preparation from postnatal mice that is enriched with PCs (Buard et al., 2010). The enrichment is accomplished by an immunopanning protocol, which selects first for L1CAM-positive cerebellar neurons and then for Thy1-positive PCs (Buard et al., 2010). The yield of L1CAM-positive cerebellar neurons per NPC1-deficient mouse was reduced

Abbreviations: EPSCs, excitatory postsynaptic currents; NPC, Niemann–Pick type C disease; PCs, Purkinje cells.

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(mean ± SD; 1.01 ± 0.76 × 10^6 cells) compared to cells isolated from wildtype littermates (1.99 ± 1.34 × 10^6 cells; 4 preparations; $p = 0.07$, Wilcoxon matched pairs test), possibly due to a lower number of granule cells. As described previously (Buard et al., 2010), cultures of L1CAM- and Thy1-selected cells comprise granule cells, GABAergic interneurons and PCs, the latter of which can be reliably recognized by the large size of their somata. After one week in defined medium, the neurons formed an extensive network of neurites (Fig. 1A). Cytochemical staining with filipin, a fluorescent antibiotic that binds to unesterified cholesterol, revealed that PCs isolated from mutant mice but not from wildtype animals showed marked intracellular staining (Fig. 1). A similar distribution was observed in primary cultures of glial cells that were prepared from NPC1-deficient mice (Fig. 1). This indicated that cultured PCs and glial cells lacking NPC1 maintain the characteristic intracellular accumulation of cholesterol that they show in vivo (Reid et al., 2004).

We next tested, whether the lack of NPC1 affected the level of synaptic activity in PCs using whole-cell patch-clamp recordings. In the absence of glial cells, PCs from wildtype and mutant mice showed a similarly low level of synaptic activity with inhibitory postsynaptic currents occurring even more rarely than excitatory postsynaptic currents (Figs. 2, 3). Based on our previous finding that glial cells strongly increase synaptic input to PCs (Buard et al., 2010), we cultured PCs with glial cells and tested whether the lack of NPC1 in glial cells affected synaptic activity in PCs. Glial cells with or without NPC1 enhanced significantly the frequency and the size of spontaneous excitatory postsynaptic currents (EPSCs) in wildtype PCs (Fig. 3). In these cocultures, the level of

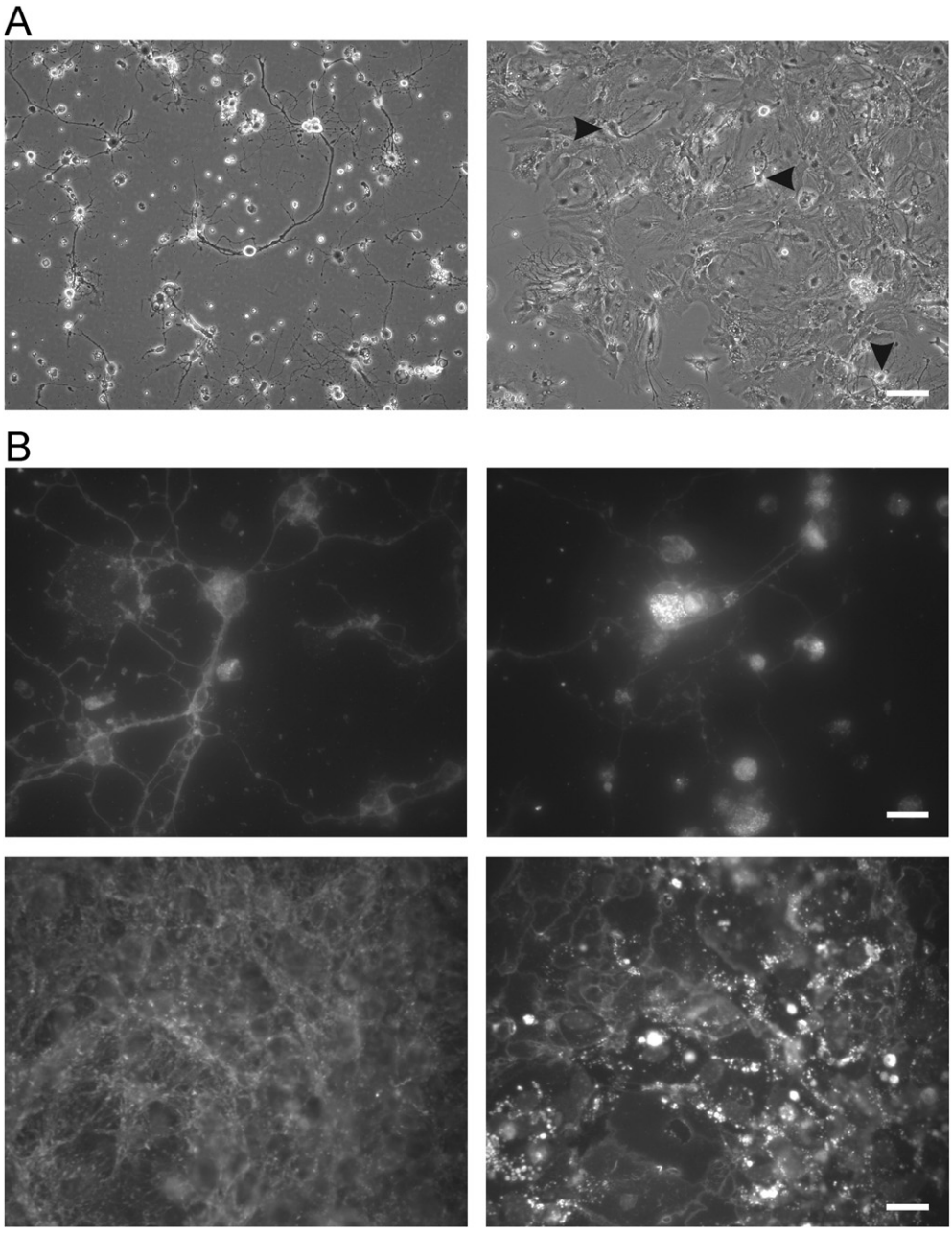

Fig. 1. Intracellular accumulation of cholesterol in cultured Purkinje cells and glial cells from NPC1-deficient mice. A, phase-contrast micrographs of PCs from wildtype mice that were enriched by immunopanning and cultured for seven days in chemically defined medium in the absence (left) or presence (right) of cerebellar glial cells. Black arrowheads indicate somata of PCs growing in coculture. Scale bar: 80 μm. B, Fluorescence micrographs of PCs (top) and glial cells (bottom) from wildtype (left) and mutant (right) mice that were cultured for one week in defined medium and then stained with filipin to reveal the distribution of unesterified cholesterol. Cells from mutant mice show intracellular accumulation of cholesterol. Scale bar top: 20 μm, bottom: 50 μm.

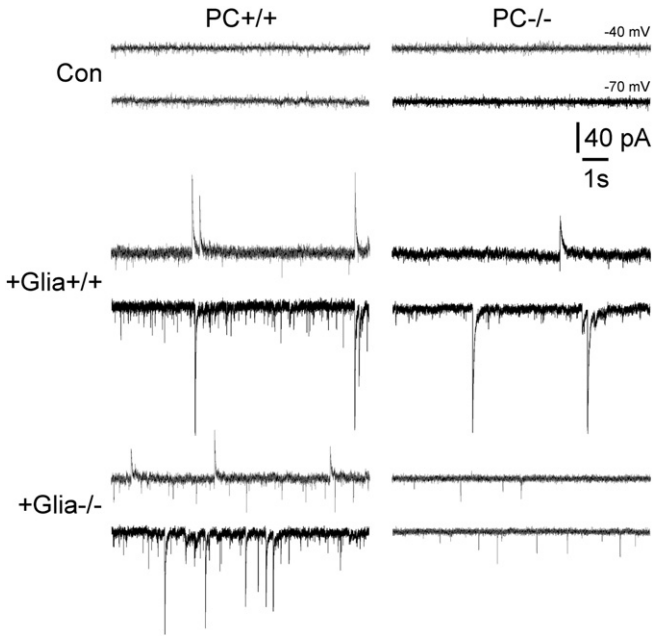

Fig. 2. Spontaneous synaptic activity in cultured PCs from wildtype and NPC1-deficient mice. Representative traces of spontaneous synaptic activity that was recorded at different holding potentials to monitor excitatory (−70 mV) and inhibitory postsynaptic currents (−40 mV) in PCs from wildtype (left) and mutant mice (right) that were cultured for seven days in the absence of glial cells (Con, top) and in the presence of glial cells that were prepared from wildtype (middle) or from mutant mice (bottom).

inhibitory synaptic activity was also elevated, but most PCs still lacked inhibitory postsynaptic currents precluding a detailed analysis. We next tested, whether glial cells could also enhance synaptic activity in PCs from mutant mice. Indeed, glial cells from wildtype animals strongly enhanced the frequency of spontaneous EPSCs in PCs from mutant mice. However, in cocultures, where both types of cells lacked NPC1, the size and frequency of spontaneous EPSCs were significantly enhanced and diminished, respectively, compared to the levels in cocultures with NPC1-expressing glial cells (Fig. 3). These results indicated that the lack of NPC1 in either neurons or glial cells does not impair excitatory synaptic activity, whereas the absence from both cell types lowered selectively the frequency of spontaneous events.

We next studied the excitability of PCs by current-clamp recordings (Fig. 4A). As shown in Fig. 4B, the resting membrane potentials of PCs from mutant mice cultured under glia-free conditions appeared more depolarized than those from wildtype mice, but the difference did not reach statistical significance ($p = 0.31$; Mann–Whitney U test). When cultured in the presence of glial cells, neurons showed similar membrane potentials regardless of the presence or absence of NPC1 in neurons or glia. Next, we measured the threshold current to elicit action potentials and the maximal frequency of action potentials that could be attained. Our results revealed no difference in the action potential-related parameters among the different culture conditions (Fig. 4) indicating that a lack of NPC1 in neurons and glial cells does not affect the excitability of PCs.

The low level of spontaneous synaptic activity in NPC1-deficient cocultures could have been caused by impaired dendritogenesis. Our previous studies showed that glia-induced formation of dendrites is a prerequisite for synaptogenesis in PCs (Buard et al., 2010) and retinal ganglion cells (Goritz et al., 2005). Immunocytochemical staining with an antibody against the dendritic protein microtubule associated protein 2 (MAP2) revealed that in cocultures, dendrite formation was significantly enhanced compared to glia-free cultures independently from neuronal or glial expression of NPC1 (Fig. 5). This indicated that the lower level of synaptic activity in NPC1-deficient cocultures was not caused by impaired dendritogenesis. Alternatively, this may have

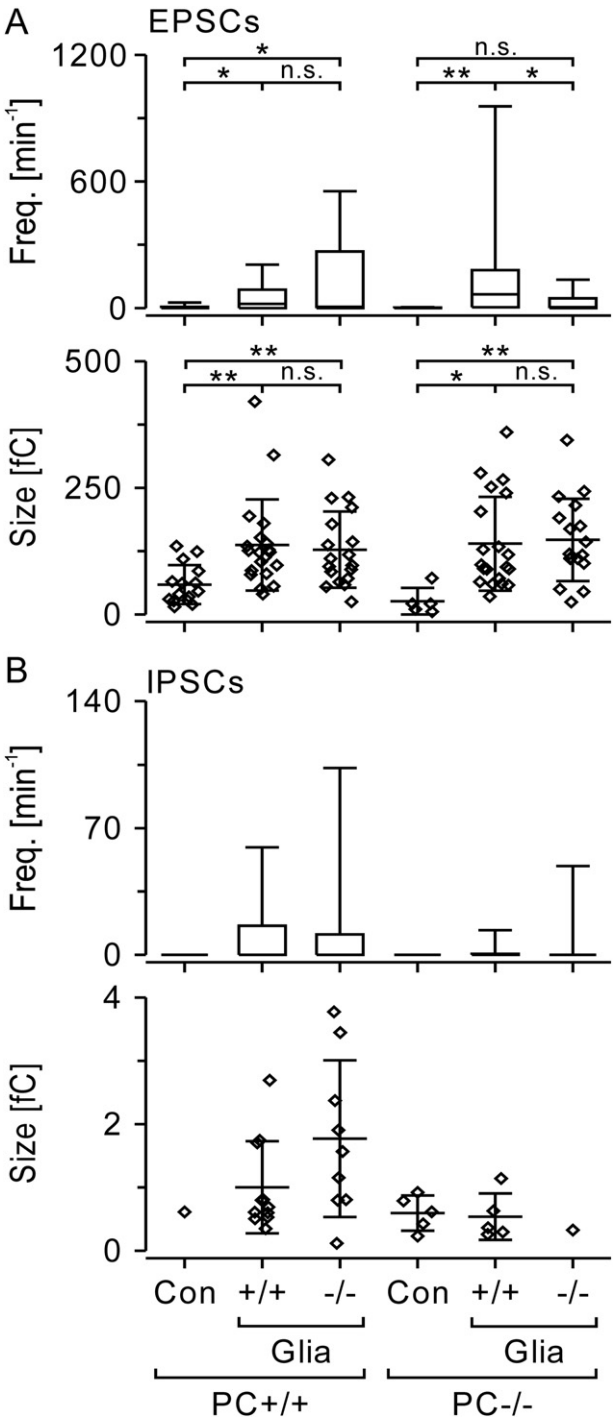

Fig. 3. Levels of spontaneous excitatory and inhibitory synaptic activity in cultured PCs from wildtype and NPC1-deficient mice. Frequency and size of spontaneous excitatory (A) and inhibitory (B) postsynaptic currents in PCs from wildtype (left) and mutant mice (right) that were cultured for seven days in the presence or absence of glial cells from wildtype and mutant mice as indicated. Coculture with glial cells enhanced excitatory synaptic activity except when glia and PCs lacked NPC1. The rare occurrence of inhibitory activity precluded further analysis ($PC^{+/+}$Con: n = 28 cells; $Glia^{+/+}$: n = 27; $Glia^{-/-}$: n = 27; $PC^{-/-}$ Con: 10; $Glia^{+/+}$: n = 24; $Glia^{-/-}$: n = 30; >3 independent culture preparations). Note that frequencies of synaptic events are displayed by box-plots and that asterisks mark statistically significant differences (Kruskal–Wallis test; 2-tailed multiple comparison), whereas n.s. indicates the absence of the latter.

been due to a reduced axodendritic synaptic input to PCs. To address this possibility, we performed immunocytochemical co-staining with antibodies against MAP2 and synaptophysin, which labels presynaptic terminals (Fig. 6A; Buard et al., 2010). Glial cells enhanced the percentage

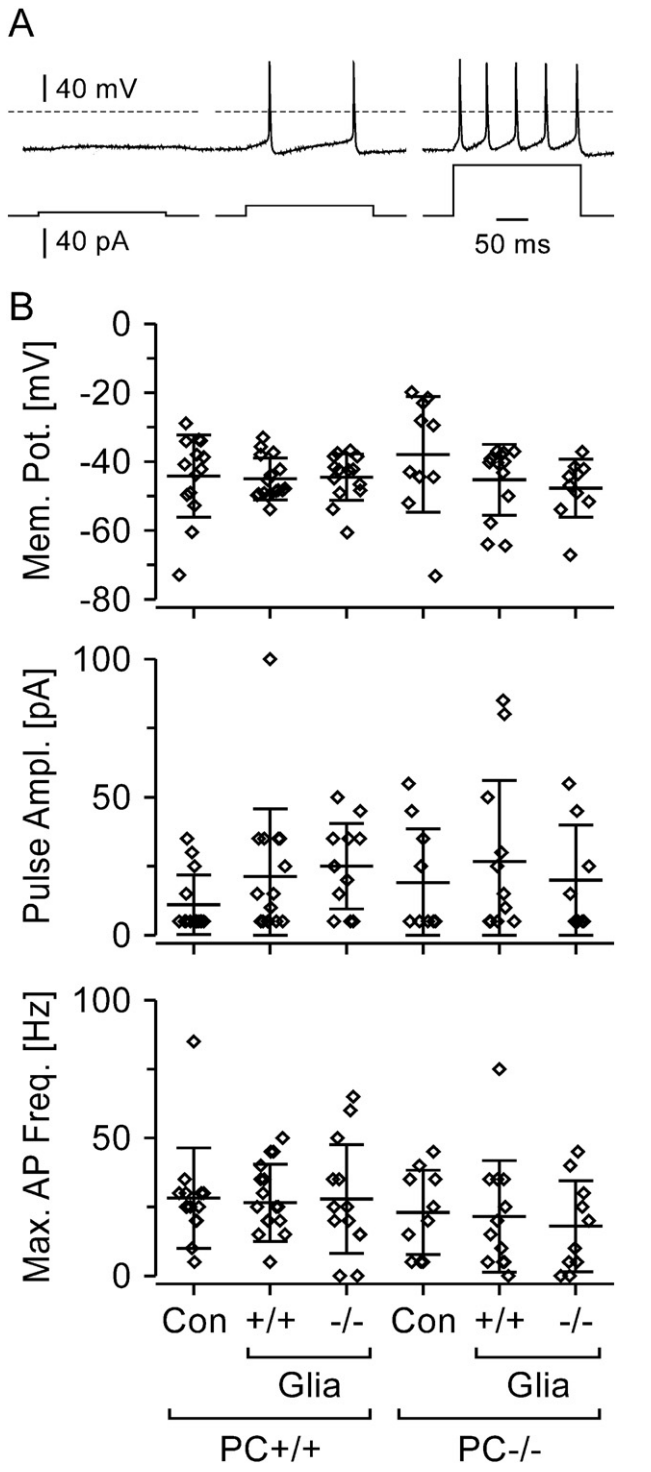

Fig. 4. Excitability of cultured PCs from wildtype and NPC1-deficient mice. A, recording traces of the membrane potential in PCs during current injection by rectangular pulses (bottom) at three selected amplitudes, which induced no AP (5 pA; left), which passed the threshold to induce APs (15 pA; middle) and which elicited the maximal frequency of APs in this cell (75 pA; right). The dashed line indicates membrane potential at 0 mV. B, resting membrane potential (top), threshold current to elicit action potentials (middle) and maximal frequency of action potentials induced by current injection (bottom) in PCs from wildtype (left) and mutant mice (right) that were cultured for seven days in the presence or absence of glial cells from wildtype and mutant mice as indicated ($PC^{+/+}$Con: n = 14 cells; $Glia^{+/+}$: n = 16; $Glia^{-/-}$: n = 14; $PC^{-/-}$ Con: 10; $Glia^{+/+}$: n = 13; $Glia^{-/-}$: n = 10; >3 independent culture preparations). None of the parameters showed statistically significant differences (Kruskal–Wallis test).

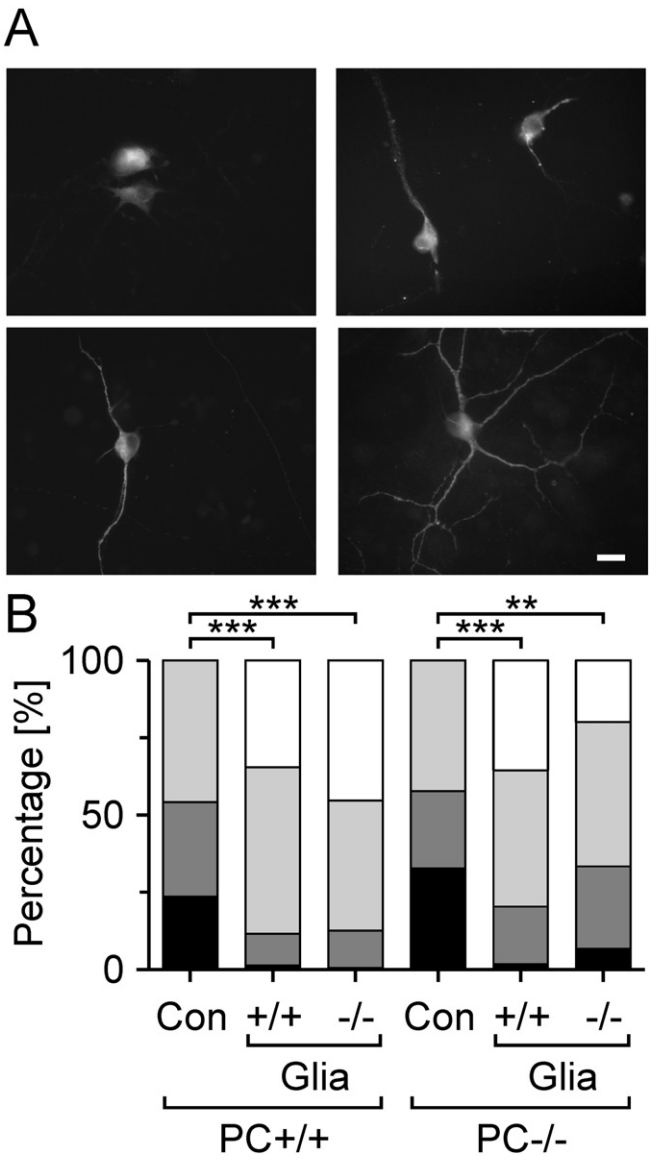

Fig. 5. Dendrite formation in cultured PCs from wildtype and NPC1-deficient mice. A, fluorescence micrographs of PCs after immunocytochemical staining for MAP2-positive dendrites. The images represent four types of PCs showing either no dendrite (top, left; coculture $PC^{-/-}$ with $Glia^{-/-}$), at least one short dendrite (top, right; $PC^{+/+}$ + $Glia^{-/-}$), prolonged dendrites (bottom, left; $PC^{-/-}$) or multiple dendrites with branches (bottom, right; $PC^{+/+}$ + $Glia^{-/-}$). Scale bar: 20 μm. B, stacked column plots showing the percentages of PCs with different degrees of dendrite differentiation. The four categories correspond to images shown in panel A (black: no dendrites; dark gray: single short; bright gray: prolonged; white: multiple branching dendrites). PCs from wildtype and mutant mice were cultured with or without glial cells from wildtype and mutant mice as indicated ($PC^{+/+}$Con: n = 85 cells; $Glia^{+/+}$: n = 78; $Glia^{-/-}$: n = 64; $PC^{-/-}$ Con: 52; $Glia^{+/+}$: n = 59; $Glia^{-/-}$: n = 30; >3 independent culture preparations). Asterisks mark statistically significant differences (Pearson's chi-square test).

of dendrites with synaptophysin-positive puncta in all types of cocultures, except for those with simultaneous NPC1 deficiency in neurons and glial cells. Under this condition, half of the PCs had dendrites without synaptophysin-positive puncta suggesting that these neurons did not receive synaptic input (Fig. 6B). This finding could explain, why nearly half of the PCs in NPC1-deficient cocultures lacked spontaneous synaptic activity (Fig. 3).

Discussion

Our study reveals that simultaneous lack of NPC1 in neurons and glial cells impairs selectively the formation of excitatory synaptic input

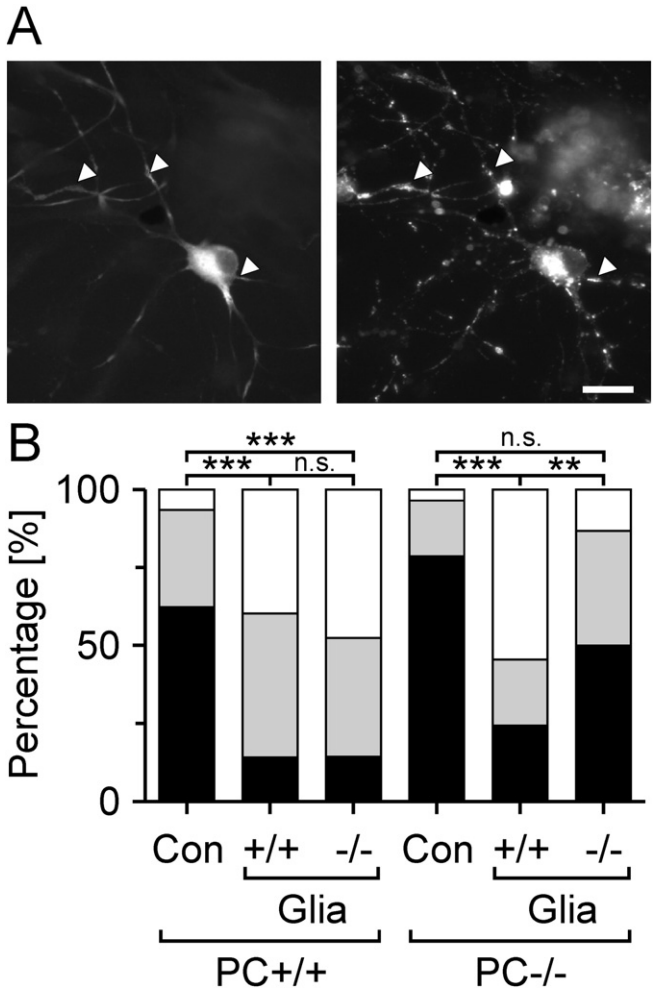

Fig. 6. Synaptic input to cultured PCs from wildtype and NPC1-deficient mice. A, fluorescence micrographs of a representative PC from mutant mice that was cocultured for seven days with glial cells from wildtype mice and then subjected to immunocytochemical staining with antibodies against MAP2 (left) and synaptophysin (right). Arrowheads indicate colocalization of the presynaptic marker and dendrites. Scale bar: 20 μm. B, percentage of PCs showing no (black), few (<5; gray) or numerous (>5; white) synaptophysin-positive puncta on MAP2-positive dendrites. PCs from wildtype and mutant mice were cultured with or without glial cells from wildtype and mutant mice as indicated ($PC^{+/+}$Con: n = 61 cells; $Glia^{+/+}$: n = 78; $Glia^{-/-}$: n = 64; $PC^{-/-}$ Con: 28; $Glia^{+/+}$: n = 33; $Glia^{-/-}$: n = 30; >3 independent culture preparations). Asterisks mark statistically significant differences (Pearson's chi-square test).

to PCs, but not their excitability or their ability to form dendrites. The absence of NPC1 from either neurons or glial cells did not affect any of these parameters.

Our study adds evidence that NPC1 deficiency affects synaptic transmission. In line with our results, the frequency of spontaneous synaptic activity was found to be lower in PCs from cerebellar slices (Bae et al., 2007) and in cultured hippocampal neurons from NPC1-deficient compared to wildtype mice (Wasser et al., 2007; Xu et al., 2011). Other studies reported enhanced synaptic activity and a block of long-term depression in PCs from adult NPC1-deficient mice (Sun et al., 2011). Evoked synaptic responses in NPC1-deficient animals were increased (Avchalumov et al., 2012; D'Arcangelo et al., 2011; Wasser et al., 2007; Zhou et al., 2011), decreased (Claudepierre et al., 2010; Phillips et al., 2008; Xu et al., 2010) or unchanged (Avchalumov et al., 2012; Deisz et al., 2005). These discrepancies may be caused by different brain areas under study and by the different experimental approaches used.

Our findings suggest a cooperative effect of neuronal and glial NPC1 on synaptic input to PCs. This effect is independent from secreted molecules like cholesterol (Karten et al., 2005; Mauch et al., 2001) or steroids (Chen et al., 2007), because glia-conditioned medium does not promote synaptogenesis in PCs (Buard et al., 2010). The selective decrease of the frequency but not of the size of spontaneous EPSCs in PCs from NPC1-deficient cocultures suggests that neuronal and glial NPC1 is required for proper presynaptic development. This is further supported by experimental evidence for a presynaptic function of NPC1 (Hawes et al., 2010; Karten et al., 2006; Sun et al., 2011; Xu et al., 2010). NPC1-deficient hippocampal neurons showed a presynaptic defect even in the absence of glial cells (Xu et al., 2011), but unlike PCs, hippocampal neurons from postnatal mice form functional synapses in vitro even in the absence of glia (Steinmetz et al., 2006). Lack of NPC1 in either neurons or glial cells left the excitability of PCs and their ability to form dendrites and excitatory synapses unaffected. This finding supports previous observations of normal electrophysiologic properties of PCs after neuron- or astrocyte-specific deletion of NPC1 (Elrick et al., 2010; Yu et al., 2011).

At present, it is unclear, whether an imbalance of synaptic input to PCs impacts their survival in vivo or after long-term culture. Some studies on transgenic mice suggest that neurodegeneration proceeds in a cell-autonomous manner. Selective ablation of NPC1 in PCs suffices to cause their demise (Elrick et al., 2010; Ko et al., 2005; Yu et al., 2011) and selective re-expression of NPC1 in PCs saves them from degeneration (Lopez et al., 2011). Notably, the degeneration of PCs also occurred, when NPC1 was eliminated in the adult stage (Yu et al., 2011) indicating an independence from developmental defects. On the other hand, it was shown that transgenic expression of NPC1 in Gfap-positive astrocytes prolongs the life-span of NPC1-deficient mice and prevents neurodegeneration (Erickson, 2013; Zhang et al., 2008). Different genetic backgrounds of the transgenic mouse models used may impact the results.

In conclusion, our study suggests that the simultaneous absence of NPC1 from neurons and glial cells impairs presynaptic input to PCs. It remains to be determined whether an ensuing imbalance of synaptic activity contributes to the age-dependent degeneration of PCs.

Experimental methods

Mice

Balb/c mice homozygous for the $Npc1^{nih}$ allele (Balb/cNctr-$Npc1^{m1N}$/J; stock # 003092, The Jackson Laboratory, Bar Harbor, Maine, USA) and wildtype littermates were used for all experiments. Experimental procedures involving animals and their care were performed in accordance with European and French regulations on animal experimentation (Directive 86/609 CEE). For genotyping, tail biopsies were prepared from three-to four-days-old pups and genomic DNA was subjected to PCR using primers (Eurogentech, Angers, France) flanking the insertion site (mp25-8F: GGTGCTGGACAGCCAAGTA and mp25-INTR3: 5′-GATG GTCTGTTCTCCCATG-3′) as described (Loftus et al., 1997).

Cell isolation and culture

Primary cultures enriched with cerebellar PCs were prepared from seven-days-old genotyped mice as described (Buard et al., 2010; Steinmetz et al., 2006). Cells from wildtype and mutant animals were isolated and cultured in parallel. Neurons were plated at 600 cells/mm^2 in a small circle (Ø 10 mm) centered on tissue culture plates (Ø 35 mm, Falcon, BD Bioscience, France) coated with 5 μg ml^{-1} poly-D-lysine (molecular weight ~ 40 kDa; Sigma) and cultured in serum-free medium (Buard et al., 2010). Cocultures with glial cells were prepared as described (Buard et al., 2010; Steinmetz et al., 2006).

Cytochemical and immunocytochemical staining

To visualize the cellular cholesterol distribution, cultured cells were fixed (4% paraformaldehyde for 30 min) and incubated for 2 h with filipin (10 μg/ml with 1% ethanol, Sigma). Filipin fluorescence was

excited by monochromatic light (356 nm, provided by a xenon-lamp and a monochromator, Polychrome Junior, TILL Photonics), fed into the epi-illumination port of an upright microscope (Axioskop II FS, Zeiss) and digitized by an air-cooled camera (Sensicam; PCO Computer Optics, Kelheim, Germany) controlled by custom-written Labview routines (National Instruments).

Immunocytochemical staining was carried out using standard procedures (Nagler et al., 2001). Cells growing on tissue culture plates were washed with phosphate buffered saline, fixed [10 min in paraformaldehyde 4% at room temperature or 7 min in ethanol at 4 °C], permeabilized with Triton X (0.1%) and blocked for 30 min in antibody buffer containing 150 mM NaCl, 50 mM Tris, 1% bovine serum albumin (Sigma A2153), 100 mM L-lysine, 0.04% sodium azide (pH 7.4) and 50% goat serum. Cells were then incubated overnight with a mouse or rabbit anti-MAP2 (1/500; Sigma) and mouse anti-synaptophysin (1:1000; Sigma). As secondary antibodies Cy2- or Cy3-conjugated goat anti-mouse or goat anti-rabbit antibodies were used (1/500; Jackson Immunoresearch Laboratories/Dianova). Fluorescence was viewed through appropriate excitation and emission filters and a 40× objective (water-immersion, n.a. 0.8, Zeiss). Images were acquired by an air-cooled monochrome CCD camera (Sensicam) controlled by custom-written Labview routines (National Instruments). Control experiments showed absence of background staining by secondary antibodies. To quantify dendrite differentiation, PCs were grouped in four categories according to the distribution of MAP2 (no dendrite: MAP2 only in somata or only; first dendrites: at least one MAP2-positive neurite of similar length as soma diameter; prolonged dendrites: MAP2-positive dendrites longer than soma diameter; fully differentiated dendrites: well-developed MAP2-positive dendrites with branches; Fig. 5A). To quantify synaptic input, cells were assigned to three categories (0: no colocalization of synaptophysin with MAP2-positive dendrites; <5: fewer than five colocalized puncta; >5: more than 5 co-localizing puncta).

Electrophysiological recordings

Whole-cell patch-clamp recordings were performed as described (Nagler et al., 2001; Goritz et al., 2005) from PCs that were recognized by their characteristic size (Buard et al., 2010). All PCs tested were electrically excitable as indicated by the presence of large voltage-activated sodium currents in response to depolarizing voltage steps. For each cell, spontaneously occurring postsynaptic currents were recorded during 3 × 1 min at −70 mV in voltage-clamp mode. Analysis of postsynaptic currents was performed automatically by custom-written Labview routines (National Instruments). The frequency of EPSCs was determined from inward currents. At −70 mV holding potential, EPSCs could be distinguished from inhibitory postsynaptic currents, which were also inwardly directed under our recording conditions, due to their faster time course. The frequency of inhibitory postsynaptic currents was determined from outward currents recorded for 3 × 1 min at −40 mV. The size of postsynaptic currents of individual cells is represented by the 90th percentile. To study the excitability of PCs, their membrane potential was recorded in current-clamp mode with injection of depolarizing current pulses of incrementing amplitude (20 pulses, 1 Hz, 200 ms length, step size 5 pA). The responses were analyzed by custom-written Labview routines.

Data representation and statistical analysis

Graphs were created by SigmaPlot 9.01 (Systat Software GmbH, Erkrath, Germany). Unless otherwise indicated, whiskers indicate standard deviation. Non-normally distributed values were represented by box-plots (horizontal line: median; lower and upper box limits: 1st and 3rd quartiles, respectively; whiskers: 10th and 90th percentile). Statistical analysis was performed using STATISTICA 12 (StatSoft Inc., Maison-Alfort, France). Statistically significant differences were detected by appropriate tests as indicated (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Acknowledgments

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